

## THE MECHANISM OF ACTION OF POLYNUCLEOTIDE PHOSPHORYLASE

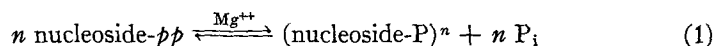
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The purpose of this paper is to review certain aspects of the mechanism of action of polynucleotide phosphorylase and to present, rather briefly, some recent findings. The discussion will be concerned with studies carried out by S. Ochoa and his associates at New York University, New York, N. Y., and with work done at the National Institutes of Health. Reference will also be made to some recent work carried out in Bethesda by Grunberg-Manago. Some of the material to be presented has already been published, but a review may be profitable at this time. Certain of the unsolved problems that face investigators in this field also will be discussed.

Polynucleotide phosphorylase was discovered by Grunberg-Manago and Ochoa in extracts of *Azotobacter agile*.<sup>1,2</sup> Studies of the nature of nucleotide incorporation into nucleic acid in *Escherichia coli* led to a recognition of the same reaction by Littauer and Kornberg.<sup>3,4,5</sup> Beers<sup>6</sup> has made extensive studies of the enzyme from *Micrococcus lysodeikticus*, and some of this work will be presented in another paper in this symposium. Olmsted<sup>7</sup> has also reported studies dealing with polynucleotide phosphorylase from *M. lysodeikticus*.

The reaction catalyzed by the enzyme may be formulated as follows:



where  $P_i$  is inorganic phosphate and nucleoside- $pp$  represents a nucleoside 5'-diphosphate. Polymers are formed in this reaction that have all of the structural features of isolated ribonucleic acid (RNA) preparations and are attacked in a similar way by hydrolytic enzymes; the experimental evidence will not be reviewed here.

The reaction can be followed in various ways. In the forward direction, one can measure the release of inorganic phosphate or the formation of acid-insoluble polymer; in the reverse direction, one can assay the rate of phosphorolysis of polymers such as RNA or adenylate polynucleotide (poly A), or of suitable oligonucleotides. Finally, one can measure the  $P_i^{32}$  nucleoside diphosphate-exchange reaction. In this assay, adenosine diphosphate (ADP) or other nucleoside diphosphate is incubated with enzyme- and  $P^{32}$ -labeled inorganic phosphate, and the ratio of these components is such that no detectable net forward reaction occurs; the amount of radioactivity incorporated into ADP is then determined.

It is of interest to see how these different assays compare quantitatively when each is performed under optimum conditions. TABLE 1 shows data for an *E. coli* fraction (first ethanol step) prepared by Hilmoie according to Littauer and Kornberg,<sup>5</sup> and for a fraction from *A. agile*<sup>8</sup> supplied by S. Mii and S. Ochoa.

The results for all of these assays are given in the same units—that is,  $\mu$ moles

per hour per milligram of protein. It is evident that measurement of  $P_i$  release in the forward reaction (EQUATION 1) gives much higher values for specific activity of the enzyme fractions than is true for the other assays. Some possible explanations for these differences will become obvious as each of the three activities of the enzyme is discussed in turn.

The forward reaction, which will be considered first, is most commonly followed by measurement of the rate of  $P_i$  formation from a single nucleoside diphosphate, or from a mixture of nucleoside diphosphates. With the enzyme preparation from *E. coli* or the earlier fractions obtained during purification of the *Azotobacter* enzyme, polymerization occurs at a linear rate until equilibrium is approached. Further, there is no stimulation, or priming, by the addition of polymers or oligonucleotides (this is in contrast to results with highly purified *Azotobacter* fraction, described below). It should be emphasized, however, that oligonucleotides of suitable structure may participate in the polymerization reaction even in cases where they afford no stimulation of enzyme activity; they are, in fact, incorporated into the polymer that is formed.

TABLE 1  
COMPARISON OF THREE ASSAYS FOR POLYNUCLEOTIDE PHOSPHORYLASE

	Micromoles per hour per milligram of protein	
	<i>E. coli</i>	<i>A. agile</i>
$P_i$ formation from ADP*	280	1000
Phosphorolysis of poly A†	20	45
ADP- $P_i^{32}$ exchange‡	35	100

\* Assay described in Mii and Ochoa.<sup>8</sup>

† Assay described in Singer.<sup>12</sup>

‡ Assay described in Grunberg-Manago *et al.*<sup>2</sup>

An example of the type of oligonucleotide incorporated is the trinucleotide, pApApA.\* This compound contains a phosphomonoester group at carbon 5' of the first adenosine residue, and the terminal nucleoside contains an unsubstituted hydroxyl group at carbon 3'. Polynucleotide phosphorylase catalyzes the addition of a mononucleotide unit to the terminal nucleoside of pApApA, and this process continues, giving a polymer chain with pApApA forming its beginning portion.

A considerable advance in our understanding of polynucleotide phosphorylase came when Mii and Ochoa<sup>8</sup> discovered a lag phase in the polymerization of ADP, inosine diphosphate (IDP), uridine diphosphate (UDP), and cytidine diphosphate (CDP), which was overcome by addition of RNA or of certain of the biosynthetic polymers. This lag period was found only with highly purified *A. agile* fractions. Singer *et al.*<sup>9</sup> found that oligonucleotides such as pApApA also overcame the lag period.† In addition to stimulating the reaction in this

\* This and other abbreviations used follow the system described in the "Instructions to Authors" in *The Journal of Biological Chemistry*, September, 1958.

† These studies were carried out with the *A. agile* fractions provided by Ochoa. Recently, the essential findings were confirmed with a fraction purified from *A. agile* by Singer and Hilmo. However, the lag period was not as striking as in the earlier work and further purification of their fraction is indicated.

manner, they were incorporated into the polymer by the same mechanism outlined above for more crude *Azotobacter* fractions.

These studies were extended to include oligonucleotides in which the hydroxyl group at C3' of the terminal nucleoside residue was blocked by a phosphomonoester residue. An example is ApApUp. With this compound (and its homologues) esterification to add new mononucleotide units and, thereby, to lengthen the chain is impossible. Consequently, oligonucleotides of this type are not incorporated, yet the surprising observation was made that they stimulate the

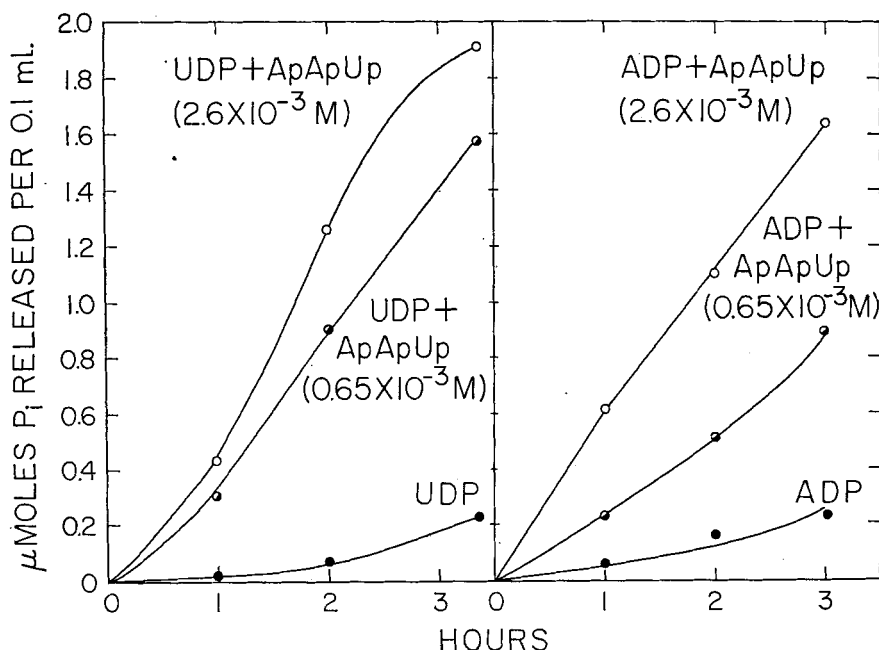


FIGURE 1. The effect of ApApUp on the lag in the polymerization of ADP and UDP with purified *A. agilis* polynucleotide phosphorylase. The reaction mixtures contained Tris buffer (pH 8.2), 7.5  $\mu$ moles; ethylenediamine tetraacetate, 0.02  $\mu$ mole; ADP or UDP, 3  $\mu$ moles in a total volume of 0.05 ml. In the ADP experiments the reaction mixtures also contained 0.5  $\mu$ mole  $MgCl_2$  and  $5.1 \times 10^{-4}$  mg. enzyme (specific activity 150 by the "exchange" assay); in the UDP experiments they contained 1.5  $\mu$ moles  $MgCl_2$  and  $1.1 \times 10^{-3}$  mg. enzyme. The concentrations of oligonucleotides are indicated on the figure. The reaction was followed by determination of the release of  $P_i$ . Incubation temperature, 37° C.

polymerization reaction, overcoming the lag period both for ADP and UDP (FIGURE 1).

There are no data to explain how these nonincorporated oligonucleotides act in overcoming the lag period. It is possible that such compounds and the various polymers stimulate the reaction in a similar fashion. However, there is no apparent specificity to be observed with oligonucleotides, while specificity relationships have been found with polymers.<sup>8</sup> The great interest in primers that are not incorporated into new chains lies in the possibility that large polynucleotide molecules of this kind may have a directing influence on the composition of the polymer synthesized.

The behavior of guanosine diphosphate (GDP) is unique because, when present alone, it cannot be polymerized by any available preparation of polynucleotide phosphorylase, whether from *E. coli* or *A. agile*. There is no reaction with a large excess of enzyme or after many hours of incubation, even with those fractions that show no lag period with other nucleoside diphosphates. With the same enzyme preparations, GDP is well utilized if mixed with other nucleoside diphosphates; thus, Grunberg-Manago *et al.*<sup>2</sup> described the preparation of poly AGUC several years ago.

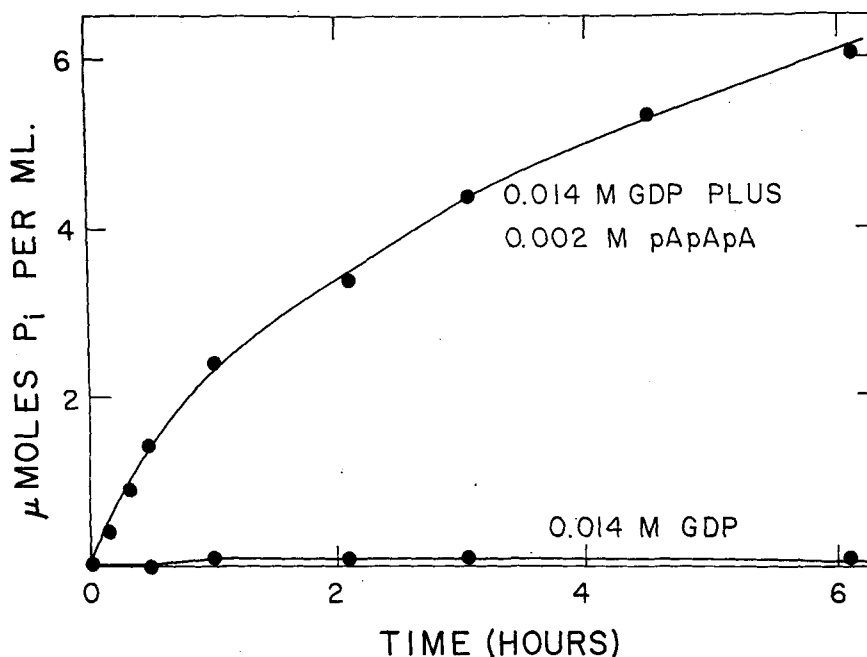


FIGURE 2. The polymerization of GDP in the presence of pApApA. The reaction mixtures contained Tris buffer (*pH* 8.2), 23  $\mu$ moles;  $MgCl_2$ , 1.5  $\mu$ moles; ethylenediamine tetraacetate, 0.06  $\mu$ mole; GDP, 2.1  $\mu$ moles; and *Azotobacter* polynucleotide phosphorylase, 0.012 mg., in a final volume of 0.15 ml. The experiment shown in the upper curve included, in addition, 0.3  $\mu$ mole pApApA. The reaction was followed by determining the release of inorganic phosphate. Incubation temperature, 37° C.

If an oligonucleotide with a free C-3' hydroxyl group is included in the incubation mixture, a polymerization reaction involving GDP does take place (FIGURE 2). An example of such an oligonucleotide is pApApA, but others would also serve. The hydroxyl group is esterified in the enzymatic reaction, forming a phosphodiester bond and adding the first guanosine monophosphate residue:



The tetranucleotide, pApApApG, is the first major product of the reaction; it has been separated by paper chromatography followed by rechromatography

in another solvent system. Hydrolysis in 1N HCl yielded adenine and guanine in a ratio of 3.2:1.0, the theoretical being 3.0:1.0. Digestion by alkali gave the expected products, adenosine 3',5'-diphosphate, adenosine 3'-phosphate, the corresponding 2'-isomers, and guanosine. Partial hydrolysis with snake venom phosphodiesterase (see Hilmeo, this monograph) gave the expected products.

FIGURE 3 illustrates the time course of the reaction. The primer, pApApA, disappears as it is incorporated. The concentration of the compound just discussed, pApApApG, first rises and then falls as the addition of guanosine

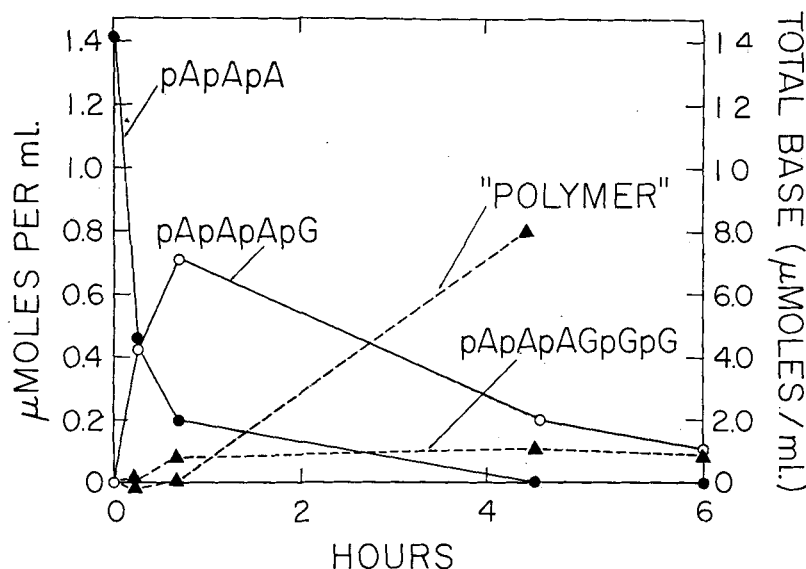


FIGURE 3. The polymerization of GDP in the presence of pApApA. The incubation mixture contained 0.08 mg./ml. of *Azotobacter* enzyme plus the following, in micromoles per milliliter: Tris buffer (pH 8.2), 150;  $MgCl_2$ , 10; ethylenediamine tetraacetate, 0.4; GDP, 13.7, and pApApA, 2. Aliquots were removed at different time intervals for quantitative paper chromatography. No polymer could be detected at 20 and 40 min; it appeared after 60 min. and increased progressively to reach a value equivalent to 8  $\mu$ moles of base per milliliter after 6½ hours. Temperature, 37° C.

monophosphate residues takes place, to give larger oligonucleotides and, finally, polymer. The polymer is nondialyzable against 0.001 M ethylenediamine tetraacetate, is precipitated by 2.5 per cent perchloric acid or 2 volumes of ethanol, and it does not migrate on paper chromatograms. Unfortunately, the amount of primer required for a reasonably rapid reaction with GDP is rather large. Thus, with 0.004 M pApApA the rate of polymerization of GDP is one fourth as fast as with ADP, and with less primer it falls off sharply. With limited amounts of enzyme it then becomes difficult to add more than about 9 guanosine monophosphate residues, on the average, per unit of primer and to do this on a reasonable scale.

In contrast to the situation with ADP and UDP, oligonucleotides such as ApApUp do not stimulate polymerization of GDP. Polymers are also inac-

tive, either because of inhibitory interactions or, perhaps, because the available concentration of terminal nucleoside residues onto which a guanylic acid residue can add is too low.

In the reverse reaction catalyzed by this enzyme, namely, the phosphorolysis of polynucleotides to form nucleoside diphosphates, poly A and poly U are rapidly attacked by polynucleotide phosphorylase.<sup>2, 5</sup> Substantial rates of phosphorolysis have been found for tobacco mosaic virus RNA, turnip yellow mosaic virus RNA, and highly polymerized yeast RNA.<sup>10</sup> On the other hand, commercial yeast RNA, which has been treated with alkali, is phosphorolyzed very slowly.<sup>5</sup>

Commercial yeast RNA is considered to be made up of relatively short chains, many of them terminated by 3'-phosphomonoester and 2',3'-cyclic phosphoryl end groups.<sup>11</sup> One possible explanation for its poor rate of phosphorolysis would be the resistance offered by such end groups. According to Singer,<sup>12</sup> oligonucleotides as large as a pentanucleotide are not phosphorolyzed if they possess the types of end group just mentioned. By contrast, oligonucleotides with a 5'-phosphomonoester end group are rapidly attacked until the molecule is reduced in size to a compound with 2 nucleoside residues. Conceivably, then, commercial RNA is resistant to enzymatic splitting because most of the chains have an unfavorable end-group structure. However, Grunberg-Manago has found that phosphorolysis of commercial RNA proceeds to the extent of at least 90 per cent.<sup>13</sup> Possibly in a molecule larger than a pentanucleotide, enzymatic break down is possible even with a 3'-phosphomonoester or 2',3'-cyclic phosphoryl end group.

In a preparation of RNA consisting of chains with an unsubstituted terminal nucleoside residue mixed with other chains in which a phosphate group is monoesterified at C-3' of the terminal residue, would chains of the second type inhibit the rate of phosphorolysis of chains that did not have such terminal phosphate groups? With this question in mind, Singer *et al.*<sup>14</sup> recently measured the rate of phosphorolysis of "5'-ended" oligonucleotides in the presence of "3'-ended" oligonucleotides. The results are shown in TABLE 2. It is apparent that ApApUp inhibits the rate of phosphorolysis of pApApA, but the effect is not great, even though a substantial amount of the "3'-ended" oligonucleotide is present. Very little information is available on the end-group structure of RNA preparations, but the possibility should be kept in mind that inhibitory effects of this kind may be operating.

A second reason for a slow rate of phosphorolysis of polynucleotides is the formation of multi-stranded chains. The interaction of poly A and poly U was first observed by Warner,<sup>15</sup> and various aspects of this subject are discussed elsewhere in this monograph. Ochoa observed that when poly A and poly U were mixed in the ratio of 1:1 the rate of phosphorolysis was considerably depressed, as compared with the rate for either polymer by itself.<sup>10</sup> Grunberg-Manago made a similar observation and also noted that phosphorolysis was suppressed almost completely with a ratio of poly A to poly U of 1:2; under these conditions, a triple-stranded chain is formed.<sup>16</sup> Her results with poly I are also of interest. This polymer is known, from the work of Rich,<sup>17</sup> to exist as a random coil in dilute salt solution and as a triple helix in 0.6 M KCl. It was noted that phosphorolysis of poly I proceeded readily in dilute salt

and was suppressed nearly completely in 0.6 M KCl, whereas poly A was equally reactive in both concentrations of salt.

At this point it is profitable to discuss a third reaction catalyzed by polynucleotide phosphorylase, namely the exchange of  $P_i^{32}$  and nucleoside diphosphate. The exact mechanism of this interesting reaction is unproved. An explanation favored by Ochoa is that the incorporation of  $P_i^{32}$  into nucleoside diphosphate results from synthesis of a small amount of polynucleotide, followed by its phosphorolysis. This is a reasonable supposition and suggests the possibility that oligonucleotides might stimulate the rate of the exchange reaction.

Recently, Singer *et al.*<sup>14</sup> studied the effect of pApA and pApApA on the rate of exchange of inorganic  $P^{32}$  with ADP and UDP; a significant stimulation was

TABLE 2  
PHOSPHOROLYSIS OF OLIGORIBONUCLEOTIDES\*

Substrate†	Addition†	Rate of phosphorolysis‡
pApApA (1.3)		18.1
pApApApA (0.8)		55.7
poly A (0.9)		7.2
pApApA (1.3)	ApApUp (1.1)	14.5
pApApApA (0.8)	ApApUp (1.1)	55.7
Poly A (0.9)	ApApUp (1.1)	6.6

\* The reaction mixture contained, in 0.125 ml.: Tris buffer (pH 8.2), 5  $\mu$ moles;  $MgCl_2$ , 0.5  $\mu$ mole;  $P_i^{32}$ , 3.05  $\mu$ moles containing 448,000 cpm (polynucleotides as indicated), and 0.002 mg. *E. coli* polynucleotide phosphorylase (first ethanol step<sup>5</sup>). After 1 hour the reactions were stopped with perchloric acid, the nucleotides adsorbed onto charcoal, and the charcoal washed free of  $P_i^{32}$  and suspended in ethanolic  $NH_3$ . Aliquots of the suspension were plated and counted.

† The numbers in parentheses are micromoles of polynucleotide per milliliter; for poly A this is expressed as adenine residues.

‡ Rates of phosphorolysis are expressed as micromoles of  $P_i^{32}$  incorporated into nucleotides per hour per milligram of enzyme.

obtained (TABLE 3). These results agree with similar data obtained by Mii and Ochoa.<sup>18</sup> The experiments shown in TABLE 3 were carried out with a preparation of *Azotobacter* enzyme provided by Ochoa, which catalyzes the polymerization of ADP and UDP only after a lag period. This lag period is overcome by concentrations of pApA and pApApA similar to those described here. The maximum stimulation observed was not large, amounting to 140 per cent.

It was hoped that more striking results might be obtained with GDP, since its polymerization shows an absolute requirement for an oligonucleotide primer. By suitable adjustment of the concentration of  $MgCl_2$  and the GDP- $P_i$  ratio it was possible to obtain a rate of  $P_i^{32}$ -GDP exchange comparable to exchange rates for ADP and UDP\* (TABLE 4). Under these conditions the rate of  $P_i^{32}$ -GDP exchange was stimulated more than threefold by  $5 \times 10^{-3}$  M pApApA. A control experiment with pApApA, but no GDP, showed a very small incorporation of  $P_i^{32}$  into nucleotide material; a correction for this was applied.

\* A detailed account of this is to be published.

To recapitulate: the enzyme preparations catalyze the polymerization of GDP only in the presence of an oligonucleotide whose configuration allows the addition of guanosine monophosphate units. Such oligonucleotides also stimulate the  $P_i^{32}$ -GDP exchange reaction. This effect is understandable if one visualizes the rapid and reversible addition of nucleotide units to the added

TABLE 3  
EFFECT OF OLIGONUCLEOTIDES ON THE ADP- $P_i^{32}$  AND  
UDP- $P_i^{32}$  EXCHANGE REACTIONS\*

Nucleotide added†	Micromoles $P_i^{32}$ incorporated into charcoal-adsorbable nucleotides, per hour, per milligram	Stimulation per cent
ADP	71	
ADP and pApA, $3 \times 10^{-3}$ M	131	85
ADP and pApApA, $5 \times 10^{-4}$ M	176	140
UDP	37	
UDP and pApA, $3 \times 10^{-3}$ M	51	38
UDP and pApApA, $5 \times 10^{-4}$ M	127	135
pApApA	6	

\* The reaction mixtures contained 10  $\mu$ moles Tris buffer (pH 8.2), 0.02  $\mu$ mole ethylenediamine tetraacetate, 0.5  $\mu$ mole  $MgCl_2$ , 0.8  $\mu$ mole inorganic  $P^{32}$  (92,000 cpm/ $\mu$ mole) in a total volume of 0.1 ml. In the ADP experiment, 0.63  $\mu$ g. and, in the UDP experiment, 1.25  $\mu$ g. of *A. agile* polynucleotide phosphorylase were added.

† Nucleotides were added in the following amounts: ADP, 0.5  $\mu$ mole; and UDP, 1.0  $\mu$ mole. Incubation was at 37° C. for 30 min.

TABLE 4  
COMPARISON OF EXCHANGE RATES WITH GDP, ADP, AND UDP\*

Substrate	Micromoles $P_i^{32}$ incorporated into charcoal-adsorbable nucleotides, per hour per milligram protein
GDP	45
ADP	51
UDP	49

\* Reaction mixtures (0.1 ml.) contained 10  $\mu$ moles Tris buffer, (pH 8.2), 0.02  $\mu$ mole ethylenediamine tetraacetate, 2.6  $\mu$ g. *A. agile* polynucleotide phosphorylase, 0.8  $\mu$ mole  $P_i^{32}$  (specific activity, 60,000 cpm/ $\mu$ mole), and one of the following combinations: 0.4  $\mu$ mole GDP and 1  $\mu$ mole  $MgCl_2$ , 0.5  $\mu$ mole ADP and 0.5  $\mu$ mole  $MgCl_2$ , or 1  $\mu$ mole UDP and 0.5  $\mu$ mole  $MgCl_2$ . After incubation for 1 hour at 37° C. the labeled nucleoside diphosphate produced in the exchange reaction was separated from inorganic  $P^{32}$  by adsorption onto charcoal and its radioactivity measured.

primer. More difficult to comprehend is the fact that a stimulation of 220 per cent was obtained with ApUp, a dinucleotide containing a 3'-phosphomonoester end group. This compound is not incorporated and does not support synthesis of poly G. This is the second example cited in this paper of stimulation of polynucleotide phosphorylase by ApUp and ApApUp. In each case, the possible mechanism is an intriguing problem for future study.

Brief reference may be made to an interesting effect of polymers on the exchange reaction. Mii and Ochoa<sup>8</sup> reported that the lag in the rate of polymerization of ADP and UDP found with purified *Azotobacter* enzyme can be



overcome by poly A and poly U, respectively. However, if the opposite pairs are used (for example, ADP and poly U), then the polymerization reaction is actually inhibited. Recently, Singer *et al.*<sup>14</sup> observed somewhat similar effects in studying the exchange reaction. Thus, when poly A, ADP, and  $P_i^{32}$  are present in a single reaction mixture, the incorporation of  $P_i^{32}$  happens to be equal to the sum of the ADP- $P_i^{32}$  exchange and the phosphorolysis of poly A, as measured separately. Similar data were obtained for the combination of UDP, poly U, and  $P_i$ ; however, when poly A is added to UDP or poly U to ADP, both exchange and phosphorolysis are inhibited. No such inhibition is

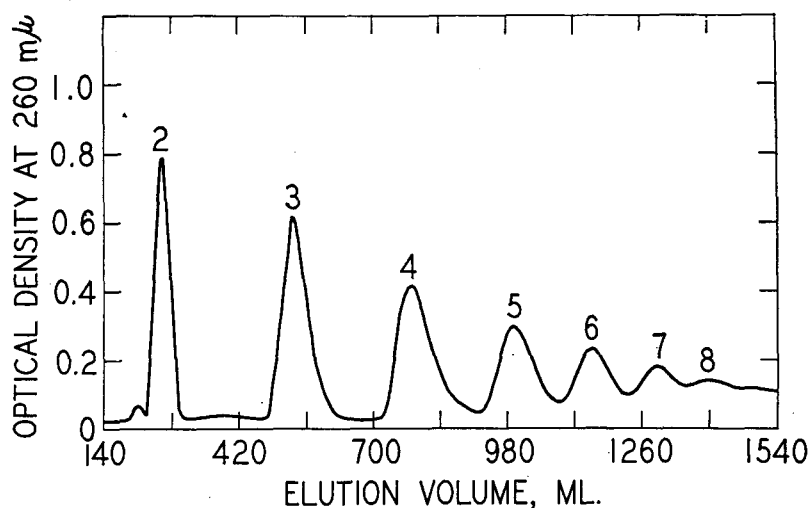


FIGURE 4. Separation of oligoribonucleotides on an "Ecteola" column. Poly AU was made by incubating equimolar quantities of ADP and UDP with polynucleotide phosphorylase; 30 mg. of the polymer was then digested with pancreatic ribonuclease and placed on a 44 × 1-cm. column of the modified cellulose adsorbent, Ecteola. Gradient elution was used, with 750 ml. of 0.015 M lithium acetate (pH 5.5) in the mixing chamber and 750 ml. of 0.4 M lithium chloride-0.015 M lithium acetate in the reservoir. Flow rate was 1.3 ml./min. The peaks represent a homologous series of oligonucleotides; thus, Peak 2 is ApUp, Peak 3 is ApApUp, Peak 4 is ApApApUp, and so on.

observed with GDP in the presence of either poly A or poly U. This is also true for thymine riboside pyrophosphate.

Brief reference should be made to methods used in the separation of the several homologous series of oligonucleotides, which have been so useful in studies of polynucleotide phosphorylase. In the past, they have been isolated by paper chromatography<sup>12</sup> and on Dowex 1-2x columns.<sup>19</sup> Recently, in Khorana's laboratory, good separations of these oligoribonucleotides were achieved by Tener and Heppel (unpublished data) using modified "Ecteola" columns.<sup>20</sup> Eluting conditions were similar to those previously employed\* for oligodesoxyribonucleotides. FIGURE 4 shows the elution diagram obtained with the homologous series beginning ApUp, ApApUp. A similar record was obtained with the series beginning pApA, pApApA.

\* See discussion in Tener *et al.*, this monograph.

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